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Bose, Sanjukta; Keller, Stephan Sylvest; Boisen, Anja; Almdal, Kristoffer

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Microcantilever Sensors for Fast Analysis of Enzymatic Degradation of Poly (D, L-lactide)

Sanjukta Bose, Stephan S. Keller, Anja Boisen and Kristoffer Almdal*

Department of Micro- and Nanotechnology, Technical University of Denmark, DK-2800 Kongens
Lyngby, Denmark

Corresponding Author

Sanjukta Bose

Ørestads Plads 345B, Department of Micro- and Nanotechnology,

Technical University of Denmark, DK-2800 Kongens Lyngby, Denmark

*Email: sbos@nanotech.dtu.dk

Telephone: +45 4525 5759

Fax: +45 4588 7762

ABSTRACT

In this work we have performed a detailed analysis of enzymatic degradation of amorphous poly (D, L-lactide) (PDLLA) by measuring the resonance frequencies of polymer coated microcantilevers before and after degradation. The miniaturized cantilever system provides a fast analysis of the biodegradation rate of PDLLA with a minute amount of sample and without the need of thermal and chemical acceleration. The degradation rate of the polymer has been estimated by multilayer cantilever theory and model simulation. A bulk degradation rate of $0.24 \mu\text{g mm}^{-2} \text{hour}^{-1}$ is estimated which agrees well with values reported in literature. The role of enzyme concentrations, pre-hydration in buffer, surface morphologies of PDLLA films and adsorption time of enzymes on the rate of degradation has been investigated. An increase in degradation rate is observed with an increase in enzyme concentration and after pre-hydration in buffer. A polymer film with a non-uniform surface degrades faster than the uniform one due to the preference of enzyme attack at film defects. A threshold time of around 3 hours is estimated for irreversible enzyme adsorption on the polymer surface after which degradation can proceed even in buffer solution in the absence of enzyme.

KEYWORDS:

Microcantilevers

Spray coating

Resonance frequency

Enzymatic degradation

PDLLA

1. INTRODUCTION

In recent years a lot of research has focused on polymers obtained from natural resources or having capability to degrade in natural environment and physiological conditions[1]. A number of polymers such as poly(lactide) (PLA), poly(glycolic acid) (PGA), poly(lactide-co-glycolides) (PLGA), polyhydroxyalkanoates (PHAs), poly(caprolactone) (PCL), and poly(butylene succinate) (PBS) have come up as biocompatible and biodegradable candidates with widespread applications in the biomedical field[2,3]. Among these biodegradable polymers PLA has gained a lot of importance due to the efficient production of its raw material, lactic acid, from sugars and starchy materials[4,5]. PLA has been used in various applications. For example, coatings on stents induce biocompatibility and eliminate immune response[6,7]. In controlled drug delivery, the therapeutic agent is incorporated into biodegradable polymeric carriers and as the polymer degrades the drug is released continuously. Therefore, the drug release kinetics can be tailored precisely by knowing the degradation profile of the polymer used[8]. Furthermore, biodegradable polymer implants and scaffolds for tissue engineering provide long term biocompatibility and eliminate the need for surgical removal[9,10]. This underlines the need for a thorough analysis of the degradation behavior of biodegradable polymers used in clinical applications.

Hydrolytic degradation plays a major role in all these applications. Hydrolytic degradation of the three stereo forms of PLA: poly(L-lactide) (PLLA), poly(D-lactide) (PDLA), and poly(DL-lactide) (PDLLA) has been studied and reported extensively over the past decades[4,5,11–16]. Hydrolysis reactions may be catalyzed by acids, bases, salts, or enzymes[10,15,17–20]. Williams first reported that hydrolysis of PLLA is catalyzed by proteinase K from *Tritirachium album*[21]. Later on, Ashley and McGinity confirmed the enzymatic degradation of PDLLA by proteinase K[22]. Thereafter enzymatic degradation of both the amorphous and the semi-crystalline form of PLA has

been studied extensively[7,17]. PDLLA has been frequently used as drug delivery film for implants and temporary scaffold for tissue engineering[10]. Degradation of PLA with proteinase K occurs typically via four steps[23]: (i) diffusion of proteinase K from the bulk solution to the PLA surface, (ii) adsorption of the enzyme on the PLA substrate, (iii) catalysis of the hydrolysis reaction i.e. the cleavage of the ester bond of PLA, and (iv) diffusion of the soluble lactic acid as degradation product from the PLA substrate to the solution. Most of the studies have used conventional methods like measuring weight loss by gravimetry, size-exclusion chromatography (SEC), differential scanning calorimetry (DSC) and X-ray diffraction (XRD) for measuring degradation of PLA. In general degradation studies require a long time[24–26] ranging from several hours to a few months and are often performed at harsh conditions of high/low pH and elevated temperatures to accelerate the experiments[27]. As biodegradable polymer films are used in many emerging biomedical applications[10,19] there is a growing need for a fast analysis of degradation behavior of polymer films at the microscopic level. In the last decade, atomic force microscopy (AFM)[28], surface plasmon resonance (SPR)[29] and quartz crystal microbalance (QCM)[7] have shown new approaches to detect biodegradation of PLA in the micro scale. Microcantilevers have already been demonstrated to be highly sensitive sensors for mechanical and thermal characterization of polymers [30]. Recently, we introduced cantilever based sensors as a tool to study enzymatic degradation of PLLA [31]. Both QCM and the micro cantilevers use the change in resonance frequency as an indicator of the degradation of a polymer film deposited on their surfaces. However, QCM measurements on polymers are typically limited to the thin film regime[7,32,33] (thickness less than 1 μ m) and characteristics exhibited by thin polymer films often strongly differ from bulk material properties[10,34]. Furthermore, QCM analysis shows some limitations for inhomogeneous polymer layers[35]. Compared to that micro cantilevers are capable of characterizing polymer films with thicknesses of several microns and varied surface morphologies

thus allowing probing macroscopic properties on a microscopic platform. In our initial experiments [31] it was concluded that coating homogeneity should be particularly improved for precise estimation of the degradation rate of the polymer.

In the present study we have used micrometer sized cantilevers to perform a considerably more detailed and systematic analysis of enzymatic degradation of thin biopolymer coatings by measuring the resonance frequency of the cantilevers before and after degradation. The miniaturized sensor system is highly sensitive towards biodegradation of minute amounts of polymer, resulting in a fast analysis of the degradation behavior without the need of thermal and chemical acceleration. Amorphous PDLLA is spray coated to form films on one side of the cantilevers which are then immersed for degradation in proteinase K in Tris-HCl buffer solution. The change in resonance frequency measured by a Laser Doppler vibrometer and the optical microscope images taken at regular time intervals reveal how the degradation of PDLLA proceeds in different conditions. The influence of varied enzyme concentrations, pre- hydration and of the time allowed for surface adsorption of enzyme on the enzymatic degradation of PDLLA is studied. By changing the spray coating parameters[36] both uniform and non-uniform PDLLA films are deposited on the silicon cantilevers and the biodegradation of films with different surface morphologies is investigated. Image analysis of the optical microscope images is conducted to monitor the dimensions of the PDLLA coating during degradation. Iterative finite element method (FEM) analysis is then carried out on a simulated cantilever model. Here, the resonance frequencies after degradation were used to determine the thickness of the polymer film, which allowed the calculation of the degradation rate.

2. EXPERIMENTAL SECTION

2.1 Materials

PDLLA (ester terminated, M_w 16,000g/mol determined by Gel Permeation Chromatography based on polystyrene calibration standards), Trizma base, Trizma HCl and dichloromethane (b. p. 40°C, ρ 1.33 g/ml at 20°C) were purchased from Sigma Aldrich. Proteinase K (recombinant, PCR grade) was obtained from Roche Diagnosis GmbH, Germany and used as received. Arrays of 8 silicon cantilevers on one chip (Octo500D), each with length $L=500\pm4\text{ }\mu\text{m}$, width $w=90\pm2\text{ }\mu\text{m}$ and thickness $t=5\pm0.3\text{ }\mu\text{m}$ were purchased from Micromotive GmbH (Mainz, Germany).

2.2 Methods

2.2.1 Spray Coating

0.5 wt% PDLLA solution in dichloromethane was used for spray coating in an Exacta Coat Ultrasonic Spraying System (Sonotek, USA) equipped with an AccuMist nozzle operated at a frequency of 120 kHz with a generator power of 1.3 W. The cantilever arrays were mounted onto a chip holder to ensure no movement of the cantilever chip during spraying. Spray coating of PDLLA was only done on the top surface of the cantilevers. The spray coating parameters were optimized to prepare uniform and non-uniform coating on the cantilever surface with an average roughness (R_a) of about $R_a = 200\text{ nm}$ and $R_a < 80\text{ nm}$, respectively[36]. For preparing uniform film, the nozzle-substrate distance was 15 mm with 10 spray-passes while for non-uniform film the nozzle-substrate distance was 45 mm with 20 spray-passes. Speed of spray of 10 mm/s, substrate temperature of 20 °C, infusion rate of PDLLA solution 0.1 ml/min and compressed nitrogen at 0.03 bar were maintained for both film types. The initial film thickness was in between 3-5 μm .

2.2.2 Enzymatic degradation

For degradation studies, each chip was placed in a petridish filled with 5 ml of 0.05 M Tris-HCl buffer solution (pH 8.6 at 23°C) containing various concentrations of proteinase K at 37°C. Resonance frequency measurement and optical microscope imaging were performed at intervals of

3 hours. For this, the chips were removed from the media, washed thoroughly with Milli-Q water to remove residues of soluble degraded products, enzymes, salts and other impurities and dried in vacuum. After characterization the chips were placed in fresh enzyme solution to restore the original condition of degradation. Alternatively, measurements were also conducted with 24 hours immersion of the cantilever chips in the degradation media. An enzyme activity assay was performed with Chromozym PL (Roche Diagnostics GmbH, Germany) to confirm no alteration of enzyme activity of proteinase K in the experimental time frame.

2.2.3 Measurement of resonance frequency

The resonance frequency (f) of the fundamental mode of a cantilever depends on the effective mass m_{eff} and the effective stiffness k_{eff} [31,37]:

$$f = \frac{1}{2\pi} \sqrt{\frac{k_{eff}}{m_{eff}}} \quad (1)$$

From (1), it is clear that the resonance frequency of the cantilever changes when a polymer layer is applied due to the change in the k_{eff}/m_{eff} ratio. In a similar way, degradation of the PDLLA coating results in a shift of f towards the reference value measured for the cantilevers before coating. For experiments performed in vacuum and assuming small cantilever deflections, the resonance frequency of a multi-layered cantilever can be approximated by[31,37]

$$f = \frac{1}{2\pi} \frac{\lambda^2}{L^2} \sqrt{\frac{\sum_i E_i I_i}{w \sum_i \rho_i t_i}} \quad (2)$$

where, L and w are the length and width, and E_i , I_i , ρ_i and t_i the Young's modulus, the area moment of inertia, the density and the thickness of the i 'th layer of the cantilever. For the first flexural mode $\lambda=1.8751$.

The resonance frequency of the first mode of the cantilevers was measured with a Laser Doppler Vibrometer (MSA-500, Polytec GmbH, Germany). The cantilever chips were placed on a piezoresistively actuated stage mounted in high vacuum at a pressure below 3×10^{-5} mbar. The

measured initial resonance frequencies of the blank cantilevers were 30 ± 5 kHz. The large deviation was due to dimensional variations of the cantilevers. Therefore in all the experiments, each cantilever was characterized individually before and after coating with PDLA to obtain the reference resonance frequencies of blank (f_b) and coated (f_c) devices respectively. For comparison of the initial shift in resonance frequency due to spray coating of polymer on the cantilevers and to accommodate for their dimensional variations, the relative resonance frequency shift (δ_0) was calculated by the following equation:

$$\delta_0 = \frac{f_b - f_c}{f_b} \times 100 \text{ [%]} \quad (3)$$

δ_0 is used as relative frequency shift at degradation time, $t_{deg} = 0$ hour in all the plots presented in this paper. Similarly, after immersion in the degradation media, at specific time intervals, the resonance frequency f_t was measured. For comparison of the shift in resonance frequency at different degradation conditions with respect to f_b , the relative resonance frequency shift (δ_t) is reported by the following equations:

$$\delta_t = \frac{f_b - f_t}{f_b} \times 100 \text{ [%]} \quad (4)$$

The subscript t stands for the time of degradation i.e., $t_{deg} = 3, 6, 9$ hours etc. All the frequency measurements were repeated three times and the average of the data is reported.

2.2.4 Image Analysis and estimation of degradation rates using FEM simulation

The surface texture of the coated cantilevers before and after degradation was observed with an optical microscope (OM) (Zeiss, Germany) in bright field mode. With the progress of degradation the dimensions of the polymer layer on the cantilever were changing such as the length, width and thickness of the coating. The change in thickness cannot be determined by the analytical model described in (2). Direct measurement of polymer thickness on cantilevers is a challenge. Therefore,

an indirect method of measuring polymer thickness by FEM simulation was chosen instead. Image J software was used on the OM images to measure the dimensions of the cantilever and the change in lengths and widths of the PDLLA coatings during degradation. These values and the measured resonance frequency of the cantilever were used to determine the thickness of the PDLLA coatings on the micro cantilevers with an iterative finite element method (FEM) using COMSOL 4.4[38]. For this purpose, the cantilever was modelled as a perfectly rectangular structure, rigidly clamped in one end (Supporting information S1). For the coated cantilevers, another block with different material properties was added on top of the cantilever (S2). For the FEM simulation, mass densities and Young's modulus of silicon and PDLLA are considered to be 2329 kg/m^3 , 1240 kg/m^3 (taken from manufacturer's data sheet) and 170 GPa, 3.2 GPa[10] respectively. It was assumed that both the modulus and the densities remain constant throughout the experiment. The eigenfrequency of a silicon cantilever with a polymer layer on the top surface was simulated for an estimated thickness of the polymer film. The obtained eigenfrequency was then compared to the measured resonance frequency and the thickness of the polymer coating was iteratively adjusted until the difference of the computed and measured frequency values were below 0.1% compared to the measured frequency. The change in width and thickness was then used to determine the degradation rates of PDLLA in proteinase K for a particular degradation condition.

3. RESULTS AND DISCUSSION

The resonance frequencies of a coated cantilever after immersion in degradation media for various time are shown in Figure 1. The dashed line in Figure 1 indicates the initial resonance frequency of the blank cantilever.

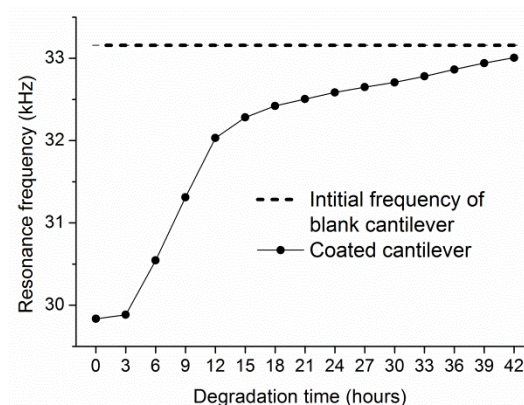


Figure 1. Resonance frequency a PDLLA coated cantilever during degradation in 50µg/ml proteinase K at 37°C. Initial frequency of the blank cantilever is shown by dashed line.

When the cantilever is coated with polymer the resonance frequency drops from 33.3 kHz to 30.1 kHz (Figure 1) due to the addition of mass on one side of the cantilever (equation (1)). When this cantilever is immersed in an enzyme solution of 50µg/ml proteinase K at 37°C, the amount of polymer on the cantilever surface decreases due to the onset of degradation and the frequency starts to increase until it approaches the initial resonance frequency of the blank cantilever.

3.1 Concentration of enzyme solution

The effect of varying concentrations of proteinase K on the degradation behavior of PDLLA is shown in Figure 2.

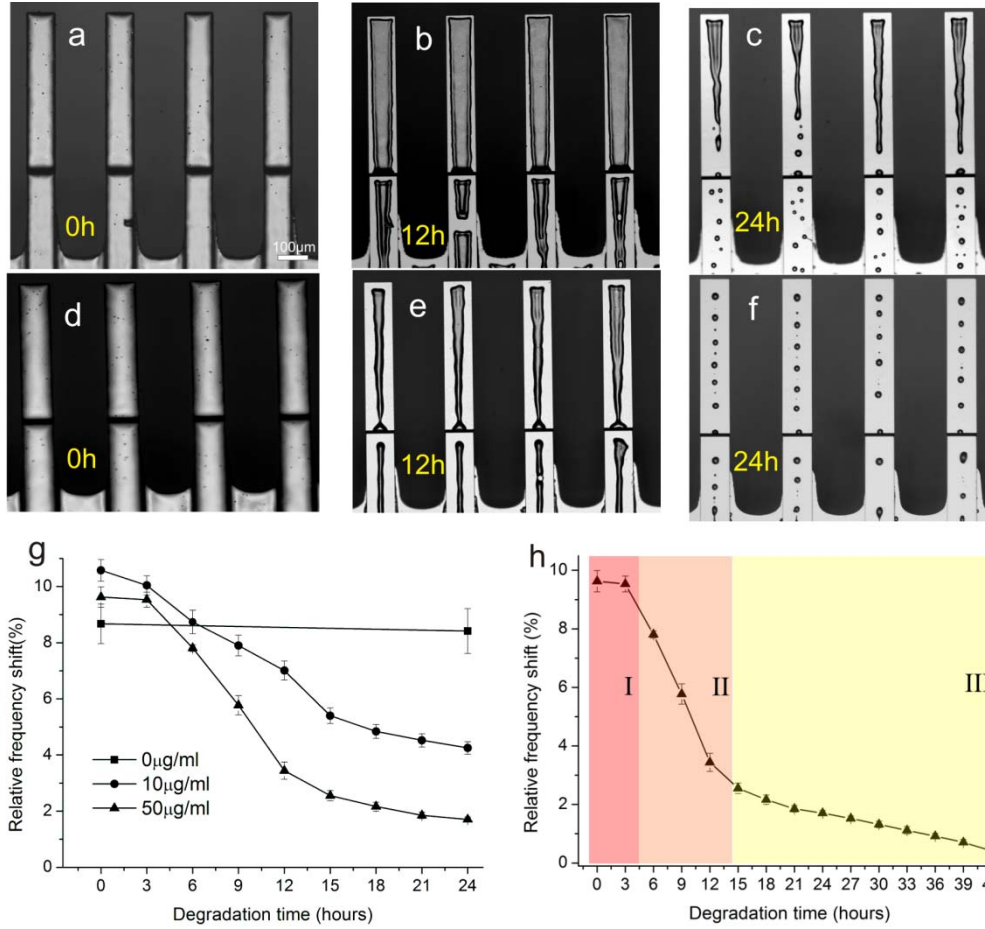


Figure 2. OM images of coated cantilevers (a-c) in 10 µg/ml proteinase K and (d-f) in 50 µg/ml proteinase K at 37 °C. Plots are shown for (g) relative frequency shift (δ_f) of coated cantilevers at different enzyme concentrations and (h) to illustrate the different regimes of degradation at 50 µg/ml enzyme concentration. The scale bar shown in (a) is valid for all the OM images.

Figure 2 shows OM images of the PDLLA coated cantilevers at specified time intervals of degradation. Comparing Figure 2 (a-c) with Figure 2 (d-f) it is clearly observed that more PDLLA is degraded from the cantilever surface in the same time interval when increasing the enzyme concentration from 10 µg/ml to 50 µg/ml. This is also reflected in the faster change in relative resonance frequency shift for the cantilevers (Figure 2g) exposed to 50 µg/ml of proteinase K compared to the ones exposed to 10 µg/ml. In the control experiment, without enzyme (0 µg/ml) in the degradation media, there is no change in frequency. The higher the concentration of enzyme in

the degradation media the higher is the amount of enzyme available to catalyze the ester hydrolysis step and hence a faster change in frequency is observed[7,32]. Figure 2h shows that three main regimes can be identified for the measurement with 50 $\mu\text{g/ml}$ of proteinase K at 37 °C.

For the first few hours of the experiment (regime I) the change of resonance frequency is very slow. It is then followed by an almost linear and relatively fast change of the resonance frequency up to ~12 hours (regime II). Then the change in frequency again slows down (regime III) and continues until the end of the measurement time frame. In regime I, the polymer coated chip is exposed to degradation media for the very first time. The polymer surface is hydrophobic and it is possible that the enzyme adsorption on the PDLLA surface before the initiation of the degradation reaction is hindered resulting in a very slow change in frequency. In regime II, a rapid decrease in the relative frequency shift indicates that the degradation is propagating very fast removing most of the polymer coating. In regime III, the polymer coating becomes discontinuous and eventually breaks into small islands of polymer (Figure 2c and 2f). Thereby the total coated area available for enzyme attack is reduced which in turn decreases the rate of relative shift in resonance frequency.

3.1.1 Regime I: Pre-hydration effects

To investigate the initial delay in regime I, coated cantilevers are immersed in Tris-HCl buffer solution for 3 hours (defined as prehydrated henceforth) before immersion in 50 $\mu\text{g/ml}$ proteinase K solution.

Figure 3 shows a comparison between the relative shift in resonance frequency observed for PDLLA with and without pre-hydration in buffer solution before enzymatic degradation.

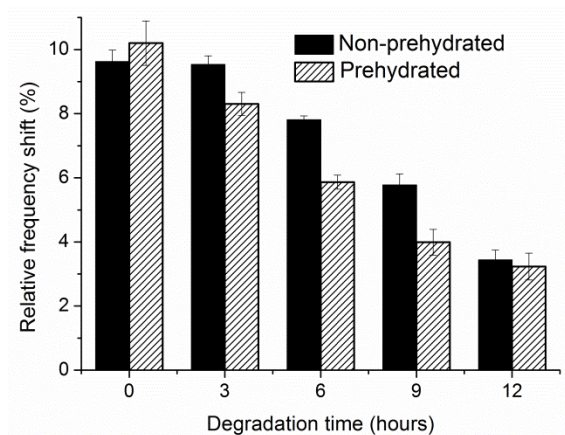


Figure 3. Relative frequency shift of prehydrated and non-prehydrated cantilevers when degraded in 50 $\mu\text{g/ml}$ proteinase K at 37 $^{\circ}\text{C}$.

From Figure 3, it is clearly observed that the cantilevers subjected to pre-hydration are showing a faster decrease in relative frequency shift (hatched columns) than the devices that are directly immersed in enzyme solution (solid columns). The result is more prominent when the first data points after 3 hours are compared in Figure 3. It can be seen that there is almost no change in relative frequency shift for non-prehydrated cantilevers while there is a considerable decrease for the prehydrated ones.

Surface hydrophilicity is considered to be one of the important factors controlling the hydrolysability of biodegradable polymers. Surface modifications like alkaline treatment, coating, and surface grafting have been used as effective methods to control the biodegradability of polymers without affecting the bulk physical properties[10,39,40]. For alkaline treatment of PLLA prior to enzymatic degradation, mostly NaOH solution (pH 12) has been used and it is reported that the cleavage of ester groups increases the number of hydrophilic hydroxyl and carboxyl groups on the surface thus increasing the surface hydrophilicity. In this study, most likely the pre-hydration for 3 hours in Tris-HCl buffer solution (pH 8.6) has modified the surface hydrophilicity of the PDLLA coating on the cantilevers which facilitates the enzyme attack when exposed to proteinase K

solution. This might explain the initial delay in regime I (Figure 2h) when the cantilevers were directly subjected to enzymatic solution without pre-hydration.

3.1.2 Regime II: Estimation of degradation rate

The steep linear decrease in resonance frequency shift in regime II is used to estimate the degradation rate of PDLA in 50 $\mu\text{g/ml}$ proteinase K at 37 °C. From OM images, the length and width of the coating is measured during degradation and FEM simulation is performed to estimate the thickness of the polymer layer as described earlier. The change in the measured width and calculated thickness of the PDLA layer is shown in Figure 4.

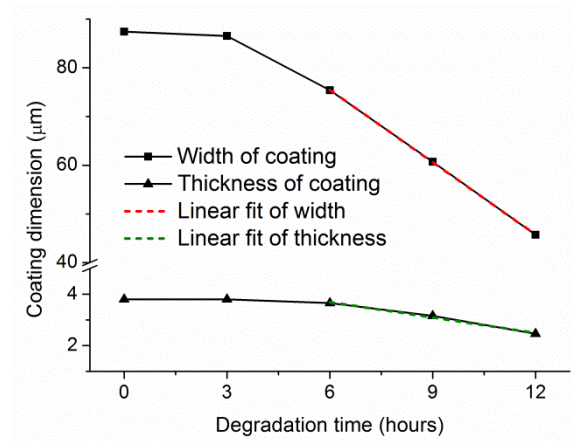


Figure 4. Change of dimension of PDLA coatings on cantilevers during degradation with 50 $\mu\text{g/ml}$ proteinase K at 37 °C.

From Figure 4, it is seen that both the width and thickness of the PDLA coating are changing simultaneously but at different rates. The dimensional change is almost negligible in regime I. In regime III, the continuity of the film is lost and simulation was omitted. The degradation rates are calculated from the slope of the curve showing the change in width and thickness in regime II .

Two different rates are observed in this study: i) A lateral degradation rate corresponding to the change in width of the film which is also visible from the OM images in Figure 2 and ii) a vertical

degradation rate corresponding to the change in thickness of the film. The lateral degradation rate is found to be $\sim 2.5\mu\text{m}/\text{hour}$ whereas the vertical degradation rate is $\sim 0.2\mu\text{m}/\text{hour}$ as calculated from the slope of the thickness plot against degradation time. The vertical degradation of the PDLLA coating is considered equal to the bulk degradation of the polymer. When calculated in terms of mass loss, the bulk degradation rate is $0.24\mu\text{g mm}^{-2}\text{ hour}^{-1}$. This agrees well with a rate of $0.28\mu\text{g mm}^{-2}\text{ hour}^{-1}$ for proteinase K degradation of PLA with 50% L-lactyl content i.e., PDLLA reported by Reeve *et al*[41]. for their specimen of dimension $2.5\text{cm}\times 1\text{cm}\times 0.06\text{mm}$ and is also in range with rates reported by others[42].

Studies have shown that in enzymatic degradation, the enzymes prefer to attack at the structural defects present on the substrate, i.e. at the edges, or in cracks and holes of the polymer films or lipid monolayers[43–46]. In our case, the enzymes seem to start fast degradation at the interface between the silicon cantilevers and the PDLLA film at the edge of the cantilevers. For small features such as in stents coated with biocompatible polymer, this lateral degradation can play a major role too. The vertical degradation rate of biodegradable polymer film is of great importance in applications in the biomedical field. Drug release kinetics can be tailored knowing the degradation rate of the polymer matrix used in drug delivery systems.

3.2 Surface morphology

The observation of high degradation rates at edges and defects indicates that the surface morphology of the PDLLA coating might have a major influence on the degradation rate. Therefore, enzyme degradation of PDLLA coatings with different surface morphologies was investigated. For this purpose, the spray coating parameters were modified to produce both uniform and non-uniform PDLLA coatings on cantilevers. Figure 5 shows the OM images and relative shift in resonance frequency with respect to the blank cantilevers for both types of coatings.

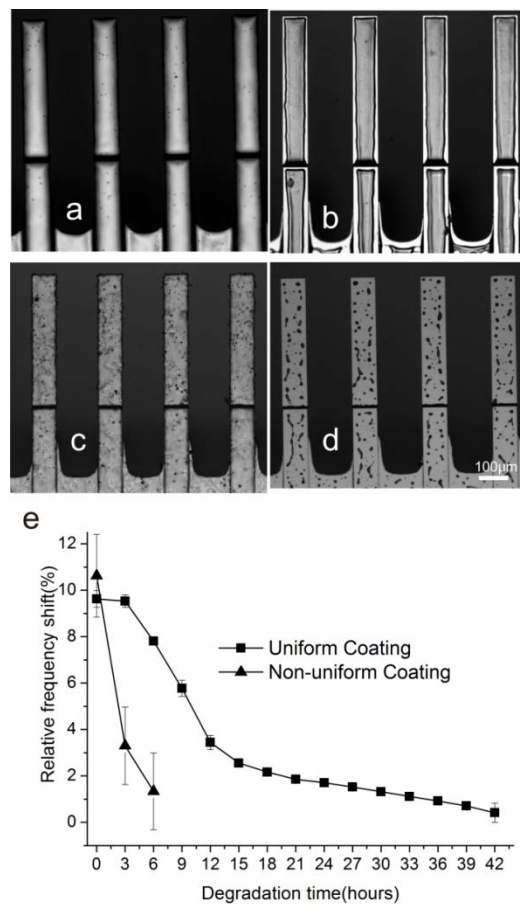


Figure 5. OM images of uniformly coated cantilevers at (a) 0 hr, (b) 6 hrs and non-uniformly coated cantilevers at (c) 0 hr and (d) 6 hrs and the relative frequency shift (e) upon degraded in 50 $\mu\text{g/ml}$ proteinase K at 37 $^{\circ}\text{C}$. The scale bar shown in (d) is valid for all the OM images.

It is seen from Figure 5 that after 6 hours there is almost a complete removal of PDLLA from non-uniformly coated cantilevers (Figure 5d) when subjected to degradation in 50 $\mu\text{g/ml}$ proteinase K. Compared to that, the uniformly coated cantilevers (Figure 5b) are still mostly covered by PDLLA exposed to the same enzyme concentration for the same time. This observation is also confirmed by the almost 6x faster shift in the resonance frequency for the non-uniformly coated devices compared to the ones coated uniformly in Figure 5e.

This confirms that enzymes show preferential attack on edges or defects in the polymer films as discussed earlier. For the non-uniform coating, the surface of the PDLLA film (Figure 5c) is

favorable for enzyme attack compared to the very smooth and intact film on the uniformly coated cantilevers (Figure 5a). In case of the uniformly coated cantilevers, degradation mainly propagates at the interface between the PDLLA film and the silicon cantilever surface at the edges of the cantilevers which acts as a defect at which the enzymes show higher ingress. The enzymes diffuse to the interfaces between the silicon and the PDLLA and catalyze the hydrolysis reaction at these interfaces which results in the lateral degradation. Similar observation has been reported by Tsuji et al. reporting selective degradation of PLLA in PCL-PDLLA blend films [47].

3.3 Influence of enzyme adsorption time

All the experiments discussed so far are conducted at the same time intervals and identical degradation conditions. Here, a new set of experiments is conducted varying the conditions for enzymatic degradation to study the influence of enzyme adsorption time. Degradation experiments are performed to find out the effect of different experimental conditions on the degradation during 24 hours as shown by the OM images and the relative frequency shift in Figure 6. Condition ‘24h E’ (Figure 6) is when a PDLLA coated cantilever chip is immersed in enzyme solution and incubated continuously for 24 hours. Condition ‘3h E+21h B’ and condition ‘1h E+23h B’ (Figure 6) are coated cantilever chips immersed in enzyme solution for 3 hours and 1 hour respectively and then transferred without washing to buffer solution for 21 hours and 23 hours respectively to see the effect of the time available for surface adsorption of enzyme on the degradation of the PDLLA film. Condition ‘24h B’ (Figure 6) is a control experiment where a coated chip is immersed in buffer solution for 24 hours to see the effect of eventual hydrolytic degradation only.

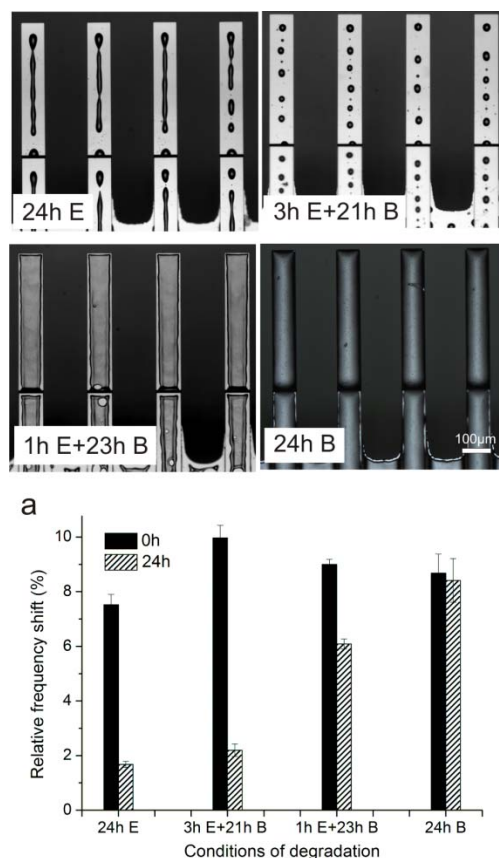


Figure 6. OM images and (a) relative frequency shift of coated cantilevers at different degradation conditions. The scale bar shown in 24h B is valid for all the OM images. Sample named 24h E is immersed in enzyme solution for 24 hours, 3h E+21h B is immersed in enzyme solution for 3 hours and in buffer solution for 21 hours, 1h E+ 23h B is immersed in enzyme solution for 1 hour and in buffer solution for 23 hours and 24h B is immersed in buffer solution for 24 hours.

From Figure 6a comparing the shift in relative frequency, conditions ‘24h E’ and ‘3h E+21h B’ have reached regime III (Figure 2h). Condition ‘1h E+23h B’ is still in regime II and condition ‘24h B’ shows an almost negligible frequency shift in 24 hours. OM images of different conditions in Figure 6 showing the PDLLA left on the cantilever surface at the end of 24 hours confirm the frequency shift findings.

Yamashita *et al.* reported the irreversible adsorption of proteinase K on the surface of PLLA films and observed that when the enzyme solution was replaced with buffer solution the PLLA film was

hydrolyzed completely without a decrease in the degradation rate[7]. Li *et al.* confirmed the observation of adsorption of enzyme on PLLA but observed no enzyme attachment on PDLA[48]. In our study, when the PDLLA coated cantilevers are exposed to enzyme solution for 3 hours followed by immersion in only buffer solution for 21 hours (Condition '3h E+21h B', Figure 6) , degradation reaction continues uninterrupted as reported by others. But for condition '1h E+23h B' when the PDLLA coated cantilevers are exposed to enzyme solution for only 1 hour followed by immersion in only buffer solution for 23 hours (Figure 6) , degradation reaction continues for few hours but ultimately stopped unlike the previous. This indicates that in the initial phase, the enzymes require some time to adsorb on the entire PDLLA layer on the cantilever surface. 1 hour as in case of condition '1h E+23h B' is too short and thus the enzymatic degradation stops after sometime. In 3 hours the enzymes have sufficient time to attach well to the PDLLA surface and continue to degrade the biopolymer when transferred to buffer solution. Condition '24h B' shows negligible effect of buffer solution on degradation of the polymer (Figure 6) proving the catalytic behavior of proteinase K in enzymatic degradation.

4. CONCLUSION

Enzymatic degradation of amorphous PDLLA has been investigated using microcantilevers. These devices are an excellent tool to do a detailed analysis of the degradation of thin PDLLA coatings by measuring the resonance frequency of the cantilever before and after degradation. The miniaturized cantilever system is highly sensitive towards biodegradation of minute amounts of polymer, resulting in a fast analysis of the degradation behavior without the need of thermal and chemical acceleration. Degradation rate of the polymer has been estimated by multilayer cantilever theory and finite element simulation where two different rates were observed for lateral and vertical degradation. Bulk degradation rate of $0.24 \mu\text{g mm}^{-2} \text{ hour}^{-1}$ was estimated which agrees well with

other reported values where larger specimen size and longer degradation time were required. We have demonstrated that the enzyme concentration, pre-hydration in buffer, surface morphologies of PDLLA films and adsorption of enzymes influence the rate of enzymatic degradation. An increase in degradation rate is observed with an increase in enzyme concentration. It has also been concluded that pre-hydration in buffer favours the overall enzymatic degradation process. A polymer film with a non-uniform surface degrades faster than the uniform one due to the preference of enzyme attack at film defects. This suggests that surface uniformity is important in biomedical applications. It was also concluded that a threshold time of around 3 hours is required for enzyme adsorption on the polymer surface after which degradation can proceed even in buffer solution. Our study helps to improve the understanding of the degradation behavior of polymer coatings. The approach can be applied to other biopolymers in different degradation conditions for a fast estimation of degradation rate.

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